

Investigation of the adrenomedullin mechanism through small interfering RNAs in human cells

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Abstract

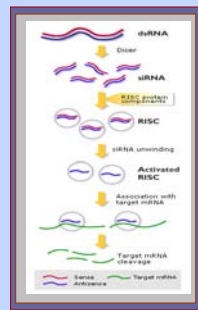
Adrenomedullin (AM) is a regulatory peptide, which inhibits Ca^{2+} -dependent agonist-stimulated aldosterone secretion and stimulates the growth of adrenocortical cells cultured in vitro. AM acts via different receptor subtypes: L1 and calcitonin receptor-like receptor (CRLR). CRLR acts as selective AM receptor only in the presence of subtypes 2 and 3 of receptor activity modifying proteins (RAMPS), which generates two AM receptors, named AM₁ and AM₂. At present, two AM-receptor antagonists are available, CGRP₈₋₃₇ e AM₂₂₋₅₂, which, however, are non-selective for the various AM receptor subtypes, because they suppress all the biological effect of AM and completely displace the tissue binding of [¹²⁵I]AM. Hence, due to the non-selectivity AM receptor-antagonist, none is known on the role played by different receptor subtypes in the mediation of the various biological effect of AM.

Small interfering RNAs (siRNAs) induced gene silencing in mammalian cells has shown great promise as a tool to suppress the expression of specific genes with high specificity. RNA interference (RNAi) is a phenomenon in which a double-stranded RNA (dsRNA) reduces the expression of the homologous gene. In vivo dsRNA molecules are reduced by the action of an endogenous ribonuclease to siRNAs and are able to specifically silencing gene expression in mammals without induction of the unspecific interferon response pathway. We have selected siRNA for human GAPD, AM, L1, RAMP2 and RAMP3. Target sequences were aligned to the human genome database in a BLAST search to eliminate those with significant homology to other genes. In the present work we investigated the effects of the suppression of AM, L1, AM₁ and AM₂ expression obtained by transfecting cells with specifically designed siRNAs on the responses of human cells cultured in vitro (fibroblasts, prostate and NCI-H295).

The siRNAs were transfected into human cells and silencing were analysed for GAPDH, AM, L1, RAMP2 or RAMP3 mRNAs. Total RNA were extracted and the expression level were determined for each experimental sample by real-time RT-PCR on the I-cycler with IQ SYBR GREEN Supermix. Our preliminary results have been show that the gene expression was decreased in all the cells studied. We are only beginning to appreciate the mechanistic complexity of this process and its biological implication. Further investigations, including the use of more human cell line, are under way to identify the specific physiological roles of AM and their receptors.

siRNA Introduction

Long double-stranded RNAs (dsRNAs; typically >200 nt) can be used to silence the expression of target genes in a variety of organisms and cell types (e.g., worms, fruit flies, and plants). Upon introduction, the long dsRNAs enter a cellular pathway that is commonly referred to as the RNA interference (RNAi) pathway. First, the dsRNAs get processed into 20-25 nucleotide (nt) small interfering RNAs (siRNAs) by an RNase III-like enzyme called Dicer (initiation step). Then, the siRNAs assemble into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs). The siRNA strands are then unwound to form activated RISCs. The siRNA strands subsequently guide the RISCs to complementary RNA molecules, where they cleave and destroy the cognate RNA (effector step). Cleavage of cognate RNA takes place near the middle of the region bound by the siRNA strand. In mammalian cells, introduction of long dsRNA (>30 nt) initiates a potent antiviral response, exemplified antiviral response can be bypassed, however, by the introduction or expression of siRNAs.



Adrenomedullin Introduction

Adrenomedullin (AM) was discovered in 1993 by the research teams headed by Drs. Eto and Matsuo in Japan (Kitamura et al., 1993). It was initially characterized as a hypotensive peptide able to elevate cAMP in platelets. The chemical structure of the new peptide was reminiscent of calcitonin gene-related peptide (CGRP) and amylin, and the three peptides are now considered members of a peptide family sharing similar biological effects and some cross-reactivity between receptors. During these years, many important discoveries have been crossed on AM biology. First of all, from being yet another vasodilator, AM has become a true multifunctional peptide with roles as varied as bronchodilator, neurotransmitter, growth factor, regulator of hormonal release, angiogenic molecule, antiapoptotic, major regulator of salt and water balance, antimicrobial molecule, among others. An important issue that has led to unexpected developments is the characterization of the receptors through which AM exerts its actions. It was realized that the AM receptor is formed by a complex requiring a seven-transmembrane domain G-protein coupled receptor called CRLR (calcitonin receptor-like receptor), and a particular single transmembrane domain protein termed RAMP (receptor activity modifying protein), which establishes ligand specificity. CRLR acts as selective AM receptor only in the presence of subtypes 2 and 3 of RAMPS, which generates two AM receptors, named AM₁ and AM₂. Several animal models have been developed in the last few years that gave us a clearer understanding of AM's functions. A transgenic mouse overexpressing AM driven by the endothelin promoter showed the protective effects of AM on septic shock. The creation of a knockout for AM resulted in embryonic lethality, indicating clearly that AM plays a major role in mammalian development. In addition, the development of other genetic approaches such as the application of adenoviruses may one day open the door for gene therapy applications. About the role of the receptor, at present two AM-receptor antagonists are available, CGRP₈₋₃₇ e AM₂₂₋₅₂, which, however, are non-selective for the various AM receptor subtypes, because they suppress all the biological effect of AM and completely displace the tissue binding of [¹²⁵I]AM. Hence, due to the non-selectivity AM receptor-antagonist, none is known on the role played by different receptor subtypes in the mediation of the various biological effect of AM. We have selected siRNA for human AM, RAMP2 and RAMP3. Target sequences were aligned to the human genome database in a BLAST search to eliminate those with significant homology to other genes. We would like investigated the effects of the suppression of AM, AM₁ and AM₂ expression obtained by transfecting cells as fibroblasts and NCI-H295.

Methods

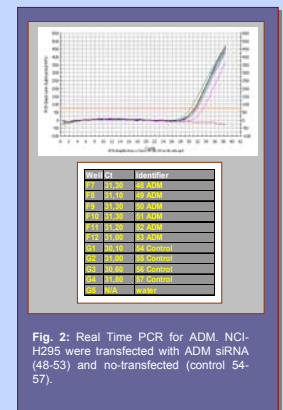
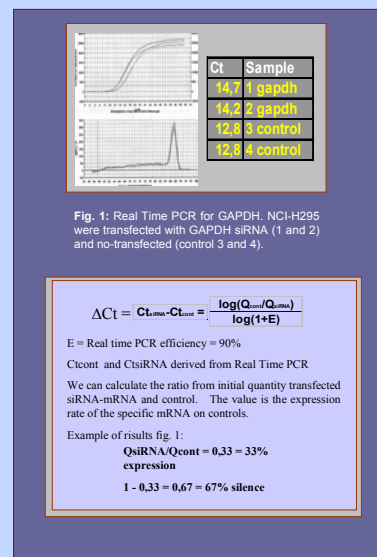
Cell Culture: NCI-H295 were propagated in RPMI medium supplemented with 2% fetal bovine serum (Sigma Aldrich), penicillin and streptomycin (Sigma Aldrich) at 37°C and 5% CO₂. Twenty-four hours prior to transfection, NCI-H295 were seeded in a 6-well plate at 2 X 10⁵ cells per well. Transfection of siRNA was carried out with siPORT™ Transfection Reagent (Ambion) as described by the manufacturer. For each well, 10-100 nM siRNA duplex was combined with 3 µl liposome in serum free RPMI and applied to cells at 50-70% confluency. Cells were assayed after 24-48 h transfection.

RNA duplex preparation: Tab. 1 shows the RNA duplex that were obtained from Dharmacon and Ambion. We used GAPDH siRNA as control and it was included into the siPORT™ Transfection kit.

Tab. 1: siRNA selected:	
Smart pool HRamp2:	CCUCAUCACUCUUGUAGUA UCAAUUGGAUCCUAUCGAA GAGAUUGCCUGGAGCACU GAGAGGAUCAUCUUGAGAG;
Smart pool HRamp3:	GGUGCAACGUCUCCGAGUU GGAAGGUUUCGACAGACAU GCAAGGTGGAGCTCTGGAA CCGAGUUAUCUGUGUACUA;
HAM-1(Ambion):	GGAUAGUCGCGCAAGCAUTT;
HAM-2(Ambion):	GGCUUAGGAGGAGGAGAAAT

RNA isolation and real time RT-PCR: Total RNA was isolated from transfected NCI-H295 using a commercially available kit according to the manufacturer's protocol (Promega). RNA was treated with DNase during the extraction (it was included into the kit) and measured prior to reverse transcription. Real time PCR was performed using specific primers.

Results



Discussion

Various siRNA duplexes were constructed against AM, RAMP2 and 3, but we have just done only preliminary study. Fig. 1 and 2 show an example of real time RT-PCR to evaluate the expression of GAPDH and AM on NCI-H295 after transfection. We have try only AM siRNA and the result do not explain the suppression of the expression of AM. It is necessary normalize every assay and we would like to do with GAPDH. More extensive studies must be conducted, in particular to optimize the transfection reaction. We would like investigated the effects of the suppression of AM, AM₁ and AM₂ expression and more work we have to do.